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TITLE: Functional Proteomics to Identify Moderators of CD8+ T-Cell Function in Melanoma

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14. ABSTRACT

In the current funding period we have performed several rounds of phage-screens to select clones that differentially bind to either tumor infiltrating cytotoxic (CD8+) lymphocytes, activated CD8+ lymphocytes from the spleen, or un-activated naïve CD8+ T cells. We have developed a high-throughput flow cytometric approach that allows us to screen the specificity of several phage clones for each of these CD8+ populations. We have identified phage that selectively bind to naïve CD8+ T cells and those that bind to both tumor-infiltrating and effector CD8+ T cells, serving as a proof of principle for the strategy. We are making modifications to 1) the phage libraries that are screened and 2) the methods by which phage clones are selected for screening in order to increase the repertoire of phage that bind to TIL selectively.

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Introduction: CD8+ T cells found within tumors are frequently dysfunctional. In many cases, dysfunction is caused by the expression of inhibitory molecules on the T cells that are designed to prevent immunopathology, but in the context of tumors, prevent T cells from performing their effector functions. Studies have shown that blocking these inhibitors with monoclonal antibodies, a process known as checkpoint blockade, can lead to the control of tumor. In melanoma patients, approximately 20% of patients make clinically relevant responses to their tumors in the presence of checkpoint blockade for the inhibitory molecule PD-1. Notably, responsiveness is strongly correlated with the expression of the ligands for PD-1. This result, while promising, begs the question why more patients do not respond. Several other inhibitory molecules have been described, suggesting that restraint of T cell function by inhibitory molecules is a multi-faceted process of critical evolutionary importance, leading to speculation that the full panoply of inhibitory molecules needs to be identified before a significantly greater proportion of patients respond to checkpoint blockade. It also raises the question as to whether different checkpoint blockade molecules are expressed by T cells that infiltrate tumors of different histology, and in different anatomical locations.
Contemporary approaches to defining inhibitory molecules have tended to depend upon defining genetic lesions that result in an autoimmune phenotype in mutant mice. Other studies have focused on using gene expression profiling to identify differentially expressed molecules that have homology to identified inhibitory molecules, or contain motifs that have previously been identified to confer inhibitory function. However, this approach suffers from uncertainty about the biology of the identified transcript. To directly indentify inhibitory molecules, we have proposed to use phage-display expression libraries to perform functional proteomics to identify molecules expressed on the surface of tumor infiltrating lymphocytes that are absent from resting or effector lymphocytes that are found in non-tumor settings. We then intend to use the phage to image the expression of these molecules in vivo, and determine the functional relevance of the molecules.
Objectives:

1. *To identify novel cell surface proteins that are differentially expressed on non-naïve tumor infiltrating T_{CD8} compared with adenovirus specific effector T_{CD8}.*
2. *To determine the contribution of identified candidates to the dysfunctional state of T_{CD8}.*
3. *To determine the ligands for molecules that can modulate the T_{CD8} functional state.*

Body:

A. Phage Screen Summary

Phage display screens were performed on tumor infiltrating lymphocytes (TILs) with the goal of finding peptide sequences specific to the TILs. These peptide sequences could then be used to develop imaging agents that are targeted to the TILs. We used an M13 phage vector, which displays a pentavalent peptide as fusions to the p3 coat protein. This system is advantageous because it allows the selection of high affinity ligands, while having the ligand DNA sequence physically linked to the ligands. By incubating the TILs with a phage library of peptide sequences, washing away low affinity phage and then eluting the bound phage, the library can be enriched for peptide sequences specific for the TILs. Following the initial round of selection the phage library can be amplified and rescreened against the same target cells to further enrich the library. This general approach was used during several screens on the TILs isolated from B16 melanoma tumors.
The 1st screen performed on TILs used NEB's Ph.D.7 library, which has a random 7mer peptide displayed on the phage. Initially, bound Ab's were removed from the TILs with a washing step, then a standard round of phage selection was performed. This entailed a 45min incubation of the phage with the TILs in suspension at 37C. After the selection, four rounds of subtraction were performed against a population of effector and naïve T Cells to remove non-specific phage. Two further rounds of amplification and selection were performed after the subtraction. Thirty clones from the final phage library were sequenced, and a lack of diversity found amongst the sequenced clones suggested that the stringency of the screen was too high. However, clones T1-1 and T1-2 are being pursued.

The 1st phage screen was modified in a 2nd phage screen that was performed on TILs reduced stringency. The stringency was reduced by changing the washing conditions prior to elution. The amount of phage library used

was also doubled. The subtraction step was also modified; four rounds were performed with all background non-TIL cells isolated from the tumor added to the effector and naïve cells. After sequencing 30 clones, a lack of diversity was also found in this screen. However, one phage (T2-1) resembled a previously identified binding sequence for VCAM-1, an important molecule for T cell extravasation into tissues. We have confirmed its capacity to bind to purified VCAM-1 (not shown) and have used this phage to optimize phage binding assays in flow cytometry (see below).

A 3rd screen was performed on the same population of TILs, with additional modifications from the 2nd to help with stringency. The screen was broken into 2 parallel screens; in one screen the Ab's were left on the cells during selection. The rationale for this was that the Ab's could be blocking nonspecific binding sites on the cell, and their blocking could increase the ratio of specific to background phage following the selection. The subtraction conditions were also changed back to conditions from the first screen, with only effector and naïve cells being used. These screens failed after the first round of amplification, possibly due to a disruption of the cell pellet during the selection or contamination during subtraction.

Screen	# of Clones Sequenced	Unique Sequences
TIL1	30	TIL1-1 TIL1-3 TIL1-38 TIL1-39
TIL2	30	TIL2-1 TIL2-2 TIL2-3 TIL2-9

A 4th screen was performed on the TILs. This screen followed the procedure of the second screen, but with modifications to decrease stringency. The incubation time was decreased for both selection and subtraction steps to decrease non-specific binding. The subtractions were performed following amplification instead of preceding it. The order was changed to prevent the inadvertent loss of phage that have high specificity and low copy number during the initial subtraction. To account for the increased number of non-specific phage that will also be present in the library as a result of this approach, the number of cells used for subtraction was increased.

A 5th screen will be performed, identical to the 4th screen except that a phage display library of 12mer displaying phage will be used instead of the 7mer library. The increased complexity of the peptide should allow for higher affinity binding to some binding sites.

Additionally, a new deep sequencing approach will be taken for sequencing the clones found in this screen after one round of selection. Using NGS in combination with phage display is an approach that was recently investiated[1]. An advantage of this approach is that a more fulsome characterization of the phage library is achieved yielding a large portion of total sequences contained in the library. Also, NGS can determine optimal sequences after one round of selection, decreasing the cost and time of completing a phage screen. We will use the Illumina MiSeq platform to sequence the libraries from the 4th and 5th TIL screens and then potentially sequence the previous screens.

B. Flow cytometric Analysis of Phage

One critical aspect that has been in need of development in order to focus on the most relevant phage clones is a flow cytometric assay that will allow discrimination of target cells that the phage bind to. We have spent considerable time optimizing the conditions for this assay, by varying the concentration of the phage, the temperature and the duration of the incubation with target cells. This is of high importance as we need an assay of high stringency and accuracy to truly define the most relevant phage for further investigation. An example of this screening is provided in Figure 1, showing relative binding of the T2-1 (also known as T1) phage to a total

lymphocyte population.

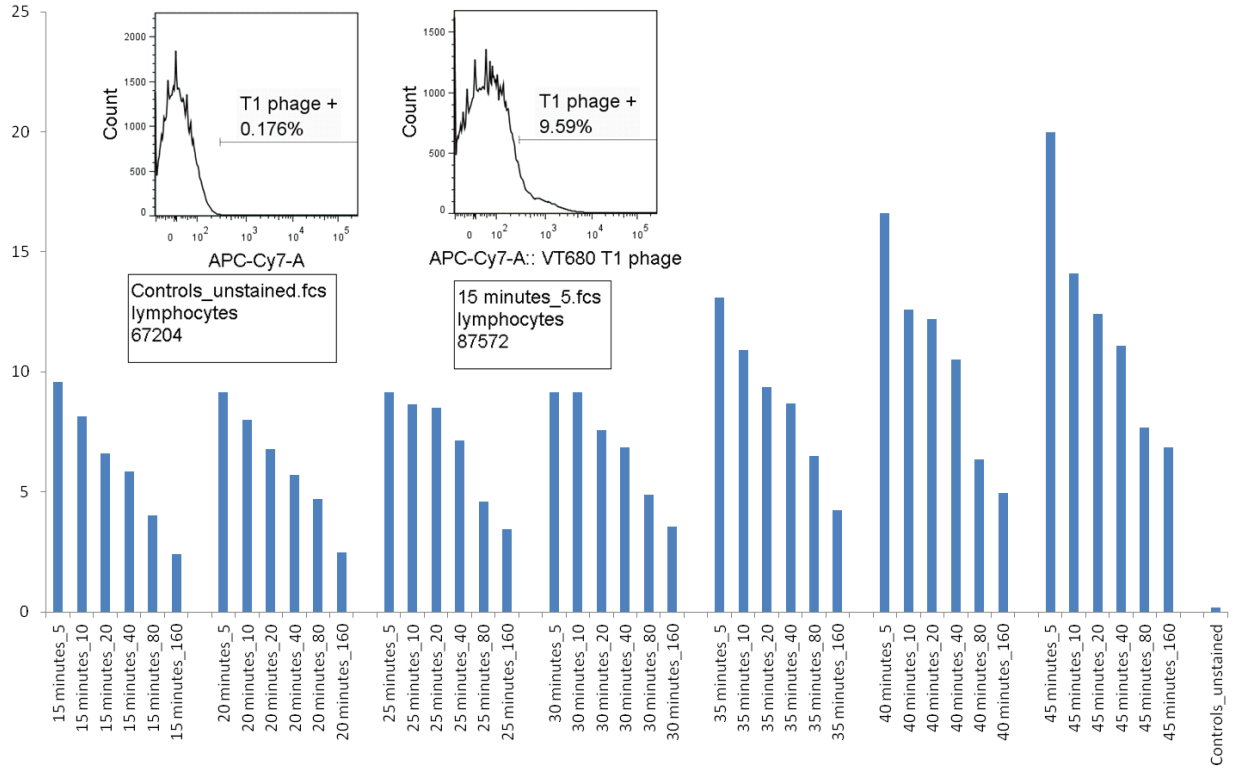

Figure 1 T2-1 phage binding screen

Once we determine the appropriate binding conditions, we have tested the selectivity of the phage by co-staining for populations of lymphocytes, to ascertain the extent to which CD8+ T cells are selectively identified (Figure 2).

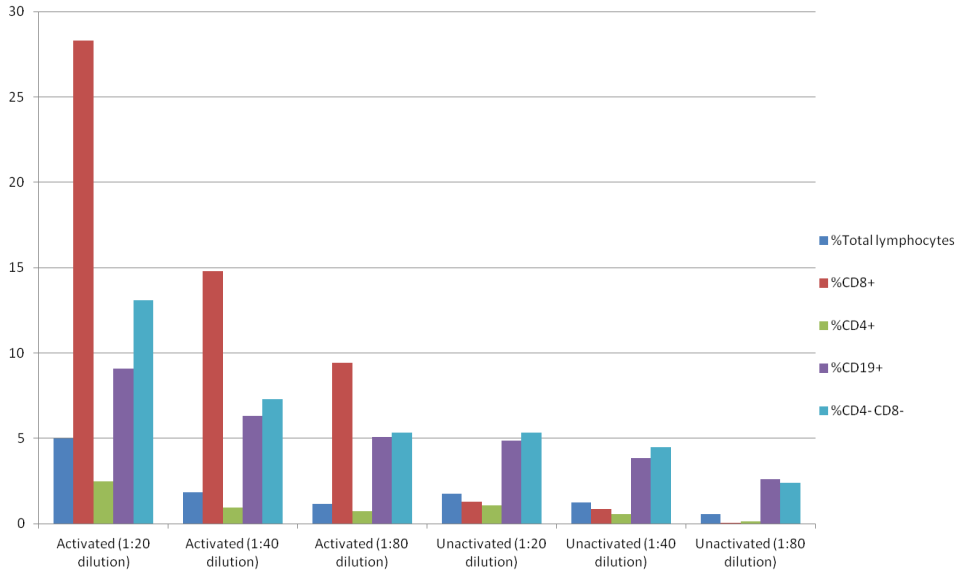

Figure 2 Selective binding of T2-1 to activated CD8 T cells

As can be seen in Figure 2, this phage binds CD8+ T cells with significantly greater frequency than other cell types, and also that binding is dependent upon the activation status of the T cells. This data serves as a good proof of principle for both the assay and also the capacity to generate phage that can selectively bind to certain populations of T cells. With this assay in place, we anticipate in the coming funding period, to be able to screen phage clones rapidly and efficiently to identify the best candidates to promote to functional assay screens.
A phage screen was also performed on the effector cells that were used in the subtraction step during the 1st screen. The amount of phage library used was doubled; so there would be an increased probability that phage specific to a binding site with low copy on the cells number would make it through the subtraction. Four rounds

of subtraction were performed against Naïve cells and then the library was amplified. Two subsequent rounds of selection were performed and then 30 clones were sequenced from the library. Nine unique clones were identified from this library and were labeled with VT680 for validation of their specificity using flow cytometry. The analysis of these clones is provided in Figure 3. As these phage each have significantly different sequences, we anticipate that they are selective for different binding partners. These effector-specific phage will be of use in FMT-based assays where we will be using the phage to identify activated T cells in vivo, and defining their ability to migrate and persist in the tumor microenvironment.

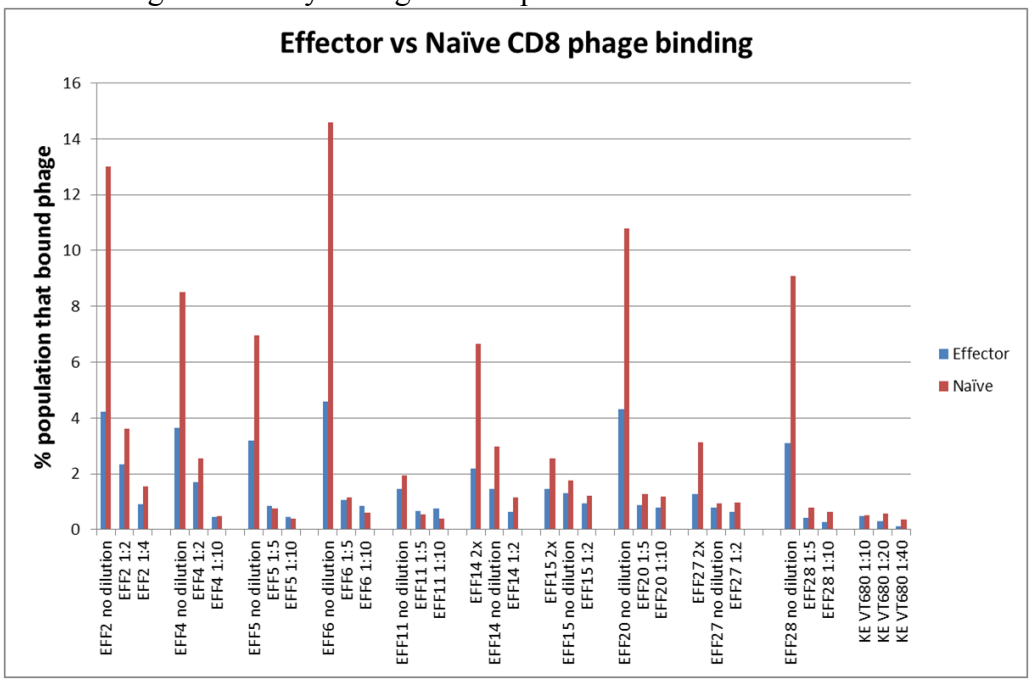

Figure 3 Screen of effector specific phage for selectivity

Based on these initial results, we are anticipating making rapid progress in year 2 of the award towards identifying a battery of TIL-specific phage and determining whether they bind to inhibitory receptors on the TIL.

Key Research Accomplishments:

1. 5 rounds of phage selection have been performed (Task 1a-1d)
2. 4 TIL-specific clones have been identified with unique sequences.
3. 9 effector specific clones with unique sequences have been identified.
4. Assay conditions for multi-parameter flow cytometric screening of phage binding have been optimized (Task 1e)
5. Selective binding of phage to activated CD8+ T cells has been demonstrated. (Task 1e)

Reportable Outcomes: N/A

Conclusion: We have made considerable progress in defining the conditions necessary for identifying phage that are specific for TIL (Task 1). We have candidate clones that are now ready for stringent assessment in our optimized flow cytometry assays, and subsequent activity in functional assays (Task 2). The limited number of clones that we have identified to date may be a consequence of challenges of working with cells directly isolated from the mouse, or may reflect that there are in fact few molecules that are selectively displayed on TIL compared to acute effector CD8+ T cells.

References

1. Peter A.C. ’t Hoen. Et. Al. Phage display screening without repetitious selection rounds. Analytical Biochemistry Volume 421, Issue 2, 15 February 2012, Pages 622–631